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Infertility of CD9-Deficient Mouse Eggs Is Reversed by Mouse CD9, Human CD9, or Mouse CD81; Polyadenylated mRNA Injection Developed for Molecular Analysis of Sperm–Egg Fusion

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CD9 is a membrane protein belonging to the tetraspanin family. Despite CD9's broad tissue distribution, the only abnormality observed in CD9-deficient mice was infertility of females, which was responsible for a defect in the sperm–egg fusion process. However, the function of CD9 in sperm–egg fusion is not clear at all because the technique to analyze the activity of molecules in sperm–egg fusion has not been established. We demonstrated that the exogenous mouse CD9, expressed by polyadenylated mRNA injection at the germinal-vesicle stage oocytes, was precisely localized to the egg plasma membrane, and the expression reversed the infertility of $CD9^{-/-}$ eggs. Then, two other tetraspanins, human CD9 and mouse CD81, overexpressed with this technique on $CD9^{-/-}$ eggs restored the fertilization rate up to ~90 and ~50% against that of wild type eggs, respectively. Moreover, in the presence of an anti-mouse CD9 mAb, which blocks sperm–egg fusion, expression of human CD9 or mouse CD81 on eggs also rescued the fusibility. These results suggested that human CD9 plays a crucial role in human fertilization, and mouse CD81 has the potential to compensate for CD9 function in sperm–egg fusion. In addition, the polyadenylated mRNA injection is effective for molecular analysis of sperm–egg fusion.

Key Words: **fertilization; sperm–egg fusion; polyadenylated mRNA injection; CD9; CD81.**

INTRODUCTION

CD9, a member of the tetraspanin family containing more than 20 molecules, has 4 transmembrane domains and 2 extracellular loops, which are characteristic of the family. CD9 and the members were reported to associate with various types of membrane proteins, including integrins, immunoglobulin superfamily proteins, and each other, and postulated to participate in cell motility, adhesion, proliferation, metastasis, viral infection, and cell fusion (Boucheix and Rubinstein, 2001). Despite the broad tissue distribution and the postulated various functions, the only abnormality observed in CD9-deficient mice was infertility of females, which was responsible for a defect in the sperm–egg fusion process (Le Naour *et al.*, 2000; Miyado *et al.*, 2000; Kaji *et al.*, 2000). In wild-type eggs, CD9 is abundantly expressed and localized on the microvilli, and an anti-mouse CD9 mAb blocks *in vitro* fertilization (Chen *et al.*, 1999). CD9 is associated with the $\alpha_6\beta_1$ integrin in eggs, which was reported to be an important molecule for fertilization (Miyado *et al.*, 2000; Almeida *et al.*, 1995). However, Miller *et al.* (2000) showed that the $\alpha_6\beta_1$ integrin was not essential for sperm–egg fusion, proven with α_6 integrin null eggs, and thus there are no molecules, other than CD9, which are clearly demonstrated to participate in sperm–egg fusion. To clarify the molecular mechanism of sperm–egg fusion, a technique for directly analyzing the activity of molecules in sperm–egg fusion is required, the functional domain of CD9 needs to be determined, and the activity of other molecules associated with CD9 must be investigated. In mammalian eggs, transcription ceases on meiotic maturation, and translation is reduced, except for mRNAs polyadenylated in the cytoplasm

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such as cyclin B1 (Seydoux, 1996; Tay *et al.*, 2000). However, by injection of the mRNAs with a long poly(A) tail (200–400 A) into the oocytes at the germinal vesicle (GV) stage, some exogenous proteins were expressed without disturbing oocyte maturation and fertilizability *in vitro*, although the technique was used only for research of maturation or activation of eggs (Vassalli *et al.*, 1989; Williams *et al.*, 1992; Aida *et al.*, 2001). In this paper, with this technique, we show that exogenous membrane proteins are abundantly expressed and precisely localized to egg plasma membrane, and we directly demonstrate that mouse CD9, human CD9, and mouse CD81 expressed on eggs from CD9-deficient mice reverse their infertility. These results demonstrate that this technique is effective for analyzing the molecules expected to participate in the sperm–egg fusion process.

MATERIALS AND METHODS

Plasmid Construction

The full-length cDNAs encoding mouse CD9 and CD81, kindly provided by Drs. M. Okazaki and H. Fujiwara, were each inserted into cloning vectors, pBluescript SK+ (Stratagene) and pPICT3, that have T7 and SP6 transcription initiation sites. Human CD9 cDNA was amplified from a human 293T cell total RNA by reverse transcription (RT)–polymerase chain reaction (PCR) and cloned into pGEM-T Easy Vector (Promega). The cDNA was sequenced to confirm fidelity.

Preparation of Transcripts and Polyadenylation

mRNAs for mouse CD9 and CD81 and human CD9 were synthesized and polyadenylated as described in Aida *et al.* (2001). Briefly, the plasmids were linearized with restriction enzymes at the 3′ ends of these cDNAs, and mRNAs were synthesized by using the T7 or SP6 MessageMachine Kit (Ambion). The transcripts were polyadenylated by incubation for 1 h at 37°C in the presence of 100 μM/liter ATP and 30 IU/ml poly(A) polymerase (Gibco) in a buffer containing 250 mM NaCl, 50 mM Tris–HCl (pH 8.1), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM dithiothreitol, 1000 IU/ml RNasin (Takara), and 0.05% BSA. To remove unincorporated ATP, the polyadenylated mRNAs were purified with an RNeasy Kit (QIAGEN) according to the manufacturer's directions, and dissolved in RNase-free distilled water. The polyadenylation, estimated to be ~200 A, was confirmed by agarose gel electrophoresis.

Oocyte Collection and Injection of RNA

CD9 homozygous deficient ($CD9^{-/-}$) and heterozygous ($CD9^{+/-}$) oocytes at the GV stage were obtained from 8- to 10-week-old mice, backcrossed with C57BL/6 mice for five generations. The genotypes of such mice were assessed by RT-PCR using primers KTP897 (5′-ACGACGGGCGTTCCTTGCGCAGCTGTG-3′) and KTP898 (5′-TCAGAAGAACTCGTCAAGAAGGCGATA-3′), which amplify the targeted locus, and KTP899 (5′-CCTGATCGTAAACCAGAGTC-3′) and KTP900 (5′-GGACACTGGAAGAGTTCATG-3′), which amplify the wild-type locus. Wild-type ($CD9^{+/+}$) oocytes at the GV stage were obtained from C57BL/6 mice for controls. To block fertilization with the anti-CD9 mAb treatment, oocytes were collected from 8- to 10-week-old ICR mice.

Immature oocytes at the GV stage were collected in M2 medium supplemented with BSA (4 mg/ml). Oocytes with cumulus cells only partially attached were selected and inserted with a micropipette for injection with 5–10 pl of poly(A)–RNA solution (300–400 ng/μl). After washing, the RNA-injected oocytes were transferred into M16 medium supplied with 5% fetal bovine serum, and cultured for 15–18 h at 37°C under 5% CO_2 in air. In Fig. 1, oocytes were collected and cultured for 3 h after injection in the presence of 100 mM 3-isobutyl-1-methlxanthine (IBMX) (Sigma).

In Vitro Fertilization

Mature eggs with a first polar body (PB1) were selected and freed from the zona pellucida by pipetting in acidic Tyrode's solution following several minutes of incubation in 0.001% chymotrypsin as described in Aida *et al.* (2001). Only the eggs that lost PB1 in this process were used to distinguish eggs with the second polar body after insemination. Spermatozoa obtained from the caudae epididymides of B6D2F1 male mice (11- to 15-week-old) were incubated in M16 medium supplemented with BSA (4 mg/ml) for more than 15 h at 37°C under 5% CO_2 in air. Eggs were inseminated by adding a small amount of the sperm suspension to the egg-containing medium. After coincubation with sperm for 2 h, eggs were washed by pipetting and fixed with 4% paraformaldehyde in PBS for 10 min. Chromosomes of eggs and sperm were stained with Hoechst 33342 to score the fertilization rates.

Immunohistochemistry and Imaging

Mouse CD9 on fixed eggs was reacted with a rat anti-mouse CD9 mAb, KMC8, or a biotinylated KMC8 (Pharmingen), followed by FITC-conjugated goat anti-rat IgG polyclonal antibodies (Cappel) or Cy3-conjugated avidin (Jackson Laboratory), respectively. Human CD9 and mouse CD81 were visualized with a mouse anti-human CD9 mAb, ALB6 (Cymbus Bio.) and a hamster anti-mouse CD81 mAb, EAT-1 (Pharmingen), followed by Cy3-conjugated donkey anti-mouse IgG polyclonal antibodies (Jackson Laboratory) and a FITC-conjugated mouse anti-hamster IgG mAb (Pharmingen), respectively. For observation of the egg surface, eggs were mounted by using the ProLong Antifade Kit (Molecular Probes). Images were captured with the Micro Max System compound Olympus fluorescence microscope. Nomarski (DIC) optics were used on all bright-light images. Overlays of captured images were processed with Adobe Photoshop 6.0.

RESULTS

Expression of CD9–EGFP Fusion Protein on Eggs by Poly(A) RNA Injection

We first tested whether a polyadenylated mRNA encoding a mouse CD9–EGFP fusion protein [mCD9—EGFP–poly(A) RNA], which was injected into oocytes at the GV stage, was translated and the resulting protein was localized to the plasma membrane. The polyadenylated mRNAs were injected into oocytes in the presence of IBMX, which maintain oocytes at the GV stage and give a longer time for RNA translation before maturation (Williams *et al.*, 1992). As shown in Fig. 1, the mouse CD9–EGFP fusion protein (mCD9–EGFP) was observed in the cytoplasm 2–3 h after

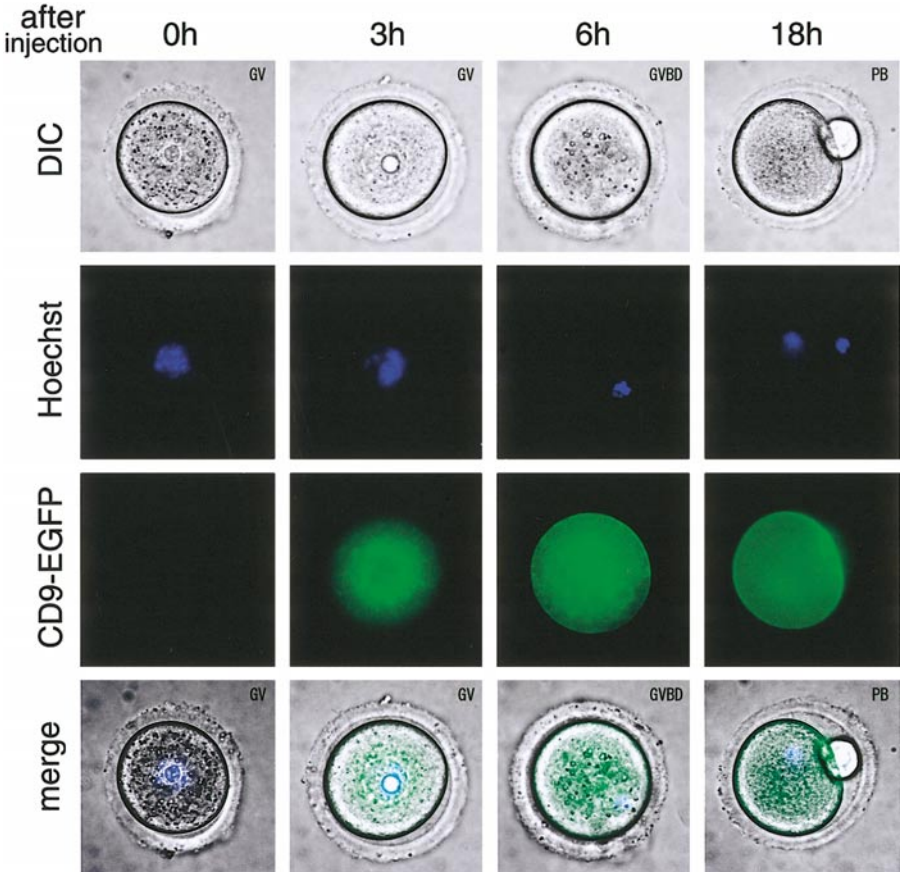

FIG. 1. Expression of CD9–EGFP on the oocytes with polyadenylated RNA injection. Immature oocytes at the GV stage were injected with CD9–EGFP RNA harboring a poly(A) tail, and cultured in the presence of 100 mM IBMX. After 3 h, oocytes were washed and cultured in the absence of IBMX to allow for meiotic maturation. Chromosomes stained with Hoechst 33342 (blue) and CD9–EGFP (green) were observed by fluorescence microscopy at the indicated time. Bottom panels show superimposed images of each upper panel. The oocyte diameter is ~70 μm. In addition to a strong signal of the surface fluorescence, a weaker signal of the intracellular fluorescence is observed in all eggs injected with mCD9–EGFP mRNA. The data shown are representative of all experiments.

injection, and then it began to localize to the plasma membrane about 6 h after injection. The localization was cleared after incubation for 18 h, and normal maturation of oocytes expressing mCD9-EGFP was observed after removal of IBMX. Because the high expression of exogenous protein was performed with or without IBMX as described by Aida *et al.* (2001), IBMX was not used in subsequent experiments.

Recovery from Infertility of CD9-Deficient Eggs by Poly(A) RNA Injection

CD9 homozygous deficient ($CD9^{-/-}$) and heterozygous ($CD9^{+/-}$) mice were previously generated (Kaji *et al.*, 2000) and were backcrossed with C57BL/6 mice for five generations. To determine whether the exogenous mCD9 expressed with this technique is functional, rescue experiments were performed by using oocytes from $CD9^{-/-}$ female mice ($CD9^{-/-}$ oocytes) (Fig. 2). $CD9^{-/-}$ oocytes at the GV stage, into which mCD9–poly(A) RNAs were injected, were matured *in vitro*, and the mature eggs were inseminated after removal of the zona pellucida. Control oocytes were prepared from $CD9^{+/+}$, $CD9^{+/-}$, and $CD9^{-/-}$ mice with no RNA injection. After coincubation with sperm for 2 h, eggs were fixed and stained with Hoechst 33342, a cell-permeant fluorescent DNA probe, and we determined the fertilization rate by counting the number of eggs released from metaphase II arrest, as a result of sperm–egg fusion. Aside from that, to judge whether the eggs were fertilized comparatively early or late after insemination, we divided fertilized eggs into two groups: eggs with the second polar body (PB2) (the percentages are shown in the black of columns in Fig. 2A) and eggs at anaphase in which eggs did not have PB2 yet (the percentages are shown in the gray). The fertilization rate (shown in the black plus the gray in the column) of the $CD9^{-/-}$ eggs with mCD9–poly(A) RNA injection was restored up to 76%, while that of $CD9^{-/-}$ eggs with no RNA injection was only 3% (Fig. 2A). This result suggested that

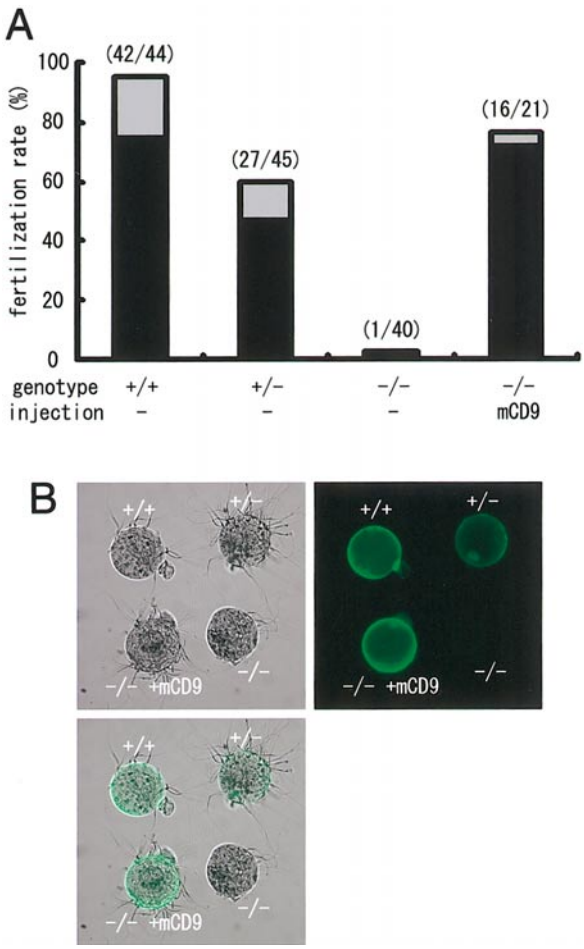

FIG. 2. Recovery of the fertilization rate of $CD9^{-/-}$ eggs by mouse CD9–poly(A) RNA injection. $CD9^{+/+}$, $CD9^{+/-}$, and $CD9^{-/-}$ oocytes at the GV stage without RNA injection (+/+, +/−, −/−) and $CD9^{-/-}$ oocytes at the GV stage with mCD9–poly(A) RNA injection (−/− +mCD9) were matured *in vitro* as described under Materials and Methods. The mature eggs, from which zona pellucida were removed, were coincubated with sperm for 2 h. (A) The fertilization rate. The numbers of fertilized eggs in total oocytes are noted above each column. The percentages of eggs at anaphase and eggs with second polar body are shown in gray and black, respectively. Experiments were done three times with more than six eggs in each group. (B) Expression of mouse CD9. Mouse CD9 (green) was stained with the anti-mouse CD9 mAb, KMC8. Each egg represents all $CD9^{+/+}$, $CD9^{+/-}$, $CD9^{-/-}$, and $CD9^{-/-}$ +mCD9 eggs used in these experiments. The lower left panel shows superimposed images of each panel. The egg diameter is ~70 μm.

mCD9 expressed by RNA injection is sufficiently functional, and this technique is useful to investigate whether other molecules can compensate for the function of mouse CD9 in sperm–egg fusion. Moreover, we found that the fertilization rate of $CD9^{+/-}$ eggs was 61%, though $CD9^{+/-}$ female mice breed similarly to $CD9^{+/+}$ female mice (Miyado *et al.*, 2000; our unpublished data). The expression level of CD9 was also examined by indirect immunofluorescence. As shown in Fig. 2B, $CD9^{-/-}$ eggs with mCD9–poly(A) RNA injection (Fig. 2B, −/− +mCD9) exhibited strong expression of CD9, the same as $CD9^{+/+}$ eggs (Fig. 2B, +/+), whereas the expression in $CD9^{+/-}$ eggs (Fig. 2B, +/−) was weaker than that in $CD9^{+/+}$ eggs. These results suggest that the fertilization rate *in vitro* correlates with the expression level of CD9.

Functional Compensation for Mouse CD9 by Human CD9 or Mouse CD81

We investigated whether two other tetraspanins, human CD9 (hCD9) and mouse CD81 (mCD81), can compensate for the function of mouse CD9. The amino acid sequence of hCD9 has about 90% homology to mCD9, but in human, the function of hCD9 in sperm–egg fusion has not yet been reported. While mCD81 is a molecule harboring about 47% homology to mCD9, its role in myoblast fusion together with mCD9 has been reported (Tachibana and Hemler, 1999). Moreover, expression of mCD81 on eggs and only small inhibition of sperm–egg fusion by anti-CD81 mAb have been reported (Takahashi *et al.*, 2001). As shown in Figs. 3A and 3B, the fertilization rate of $CD9^{-/-}$ eggs with polyadenylated hCD9 mRNA [hCD9–poly(A) RNA] injection or mCD81 mRNA [mCD81–poly(A) RNA] injection was 81 or 47%. Strong expression of hCD9 or mCD81 on $CD9^{-/-}$ eggs with RNA injection was observed at a level similar to that of endogenous mCD9 on $CD9^{+/+}$ eggs (Figs. 3C, −/− +hCD9, red; and Fig. 3D, −/− +mCD81, green). The expression of endogenous mCD81 on eggs was slightly detected with an anti-mCD81 mAb, EAT-1, or EAT-2, but this signal could not be captured well during the proper exposure time (Fig. 3D, lower left, +/+ and −/−). These staining results were consistent with the data of RNA expressions obtained from RT-PCR of mouse oocytes at the GV stage (date not shown) and with the report in which the high expression of CD9 but not of CD81 was detected in a serial analysis of gene expression (SAGE) in the human oocyte (Neilson *et al.*, 2000). Overexpressed hCD9 and mCD81 molecules seemed to be localized on the egg microvilli, which surrounded the sperm head before fusion, like endogenous mCD9 in wild type eggs as previously reported (Figs. 3E and 3F, arrows) (Kaji *et al.*, 2000). These results suggest that hCD9 and mCD81 can function like mCD9 in mouse sperm–egg fusion, although in the case of mCD81, the endogenous expression level is much lower than that of mCD9, and the activity is also lower than that of mouse or human CD9.

Recovery from Anti-mouse CD9 mAb-Induced Infertility by Expression of Human CD9 or Mouse CD81

As well as the infertility of $CD9^{-/-}$ eggs, the blocking of sperm–egg fusion by the anti-mCD9 mAb KMC8 *in vitro* has been reported (Chen *et al.*, 1999; Miller *et al.*, 2000). Thus, we examined whether this block can be reversed by

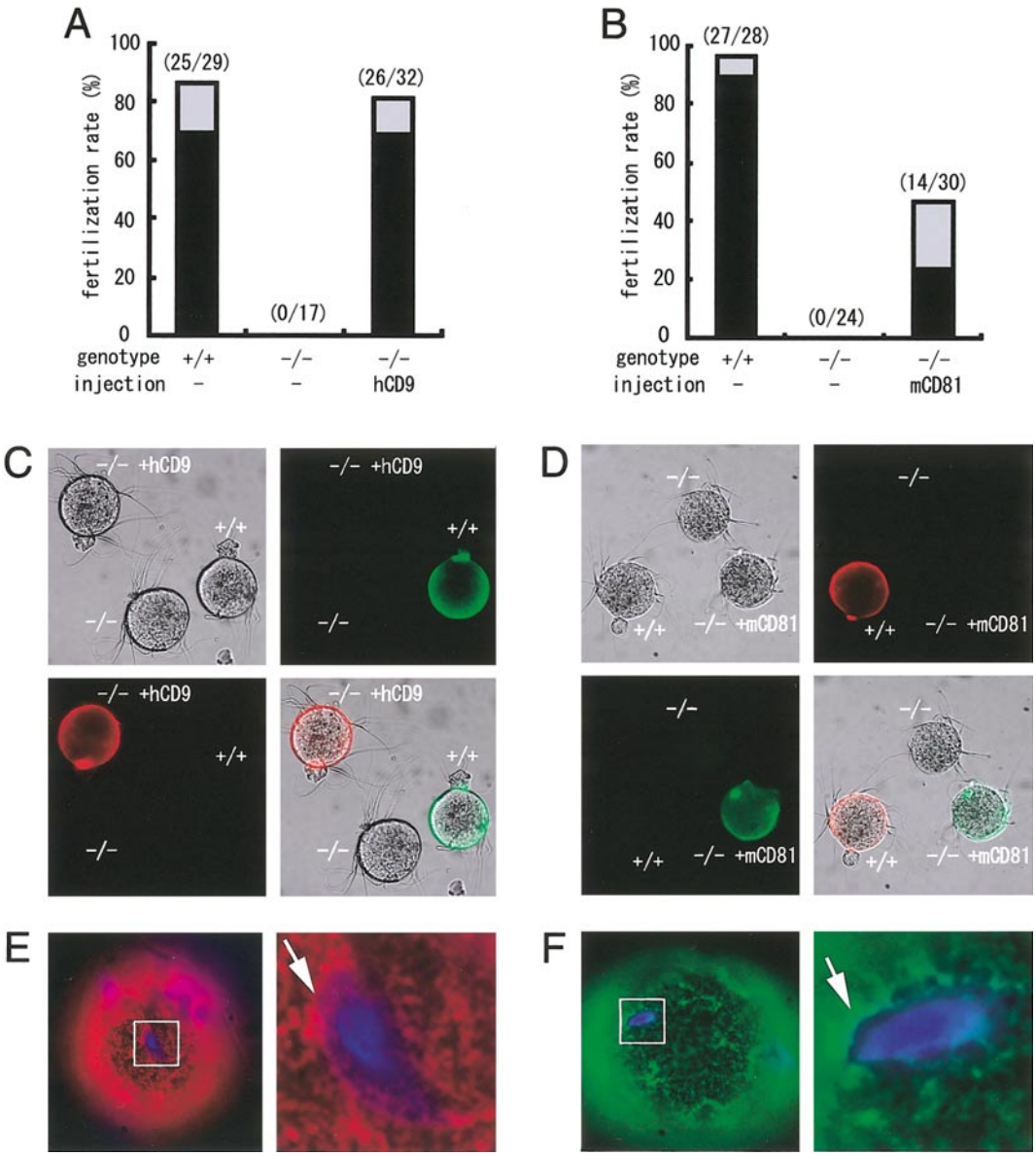

FIG. 3. Recovery of fertilization rate of $CD9^{-/-}$ eggs by overexpression of human CD9 or mouse CD81. $CD9^{+/+}$ and $CD9^{-/-}$ eggs as well as $CD9^{-/-}$ eggs expressing human CD9 or mouse CD81 were obtained as described under Materials and Methods. After removal of zona pellucida, eggs were coincubated with sperm for 2 h. The fertilization rates of eggs overexpressing human CD9 (A) and mouse CD81 (B). The numbers of fertilized eggs in total eggs are noted above each column. The percentages of eggs at anaphase and of eggs with second polar body are shown in gray and black, respectively. Experiments were done three times with more than five eggs in each group. Expression of human CD9 (C) and mouse CD81 (D). In (C), mouse CD9 (green) and human CD9 (red) were stained with the rat anti-mCD9 mAb and the mouse anti-hCD9 mAb, followed by FITC-conjugated anti-rat IgG and Cy3-conjugated anti-mouse IgG polyclonal antibodies, respectively. In (D), mouse CD9 (red) and mouse CD81 (green) were stained with the biotinylated anti-mCD9 mAb and the hamster anti-mCD81 mAb, followed by Cy3-conjugated avidin and an FITC-conjugated anti-hamster IgG mAb, respectively. (E, F) Localization of overexpressed human CD9 and mouse CD81 on the egg microvilli. Expression of human CD9 (E, red) and mouse CD81 (F, green) on the surfaces of eggs was observed after mounting with the ProLong Antifade Kit. Chromosomes of sperm (blue) were stained with Hoechst 33342. The right panels are magnified images of the white square regions in the left. Human CD9 and mouse CD81 were localized surrounding the spermhead (arrows). The staining data shown are representative of all experiments.

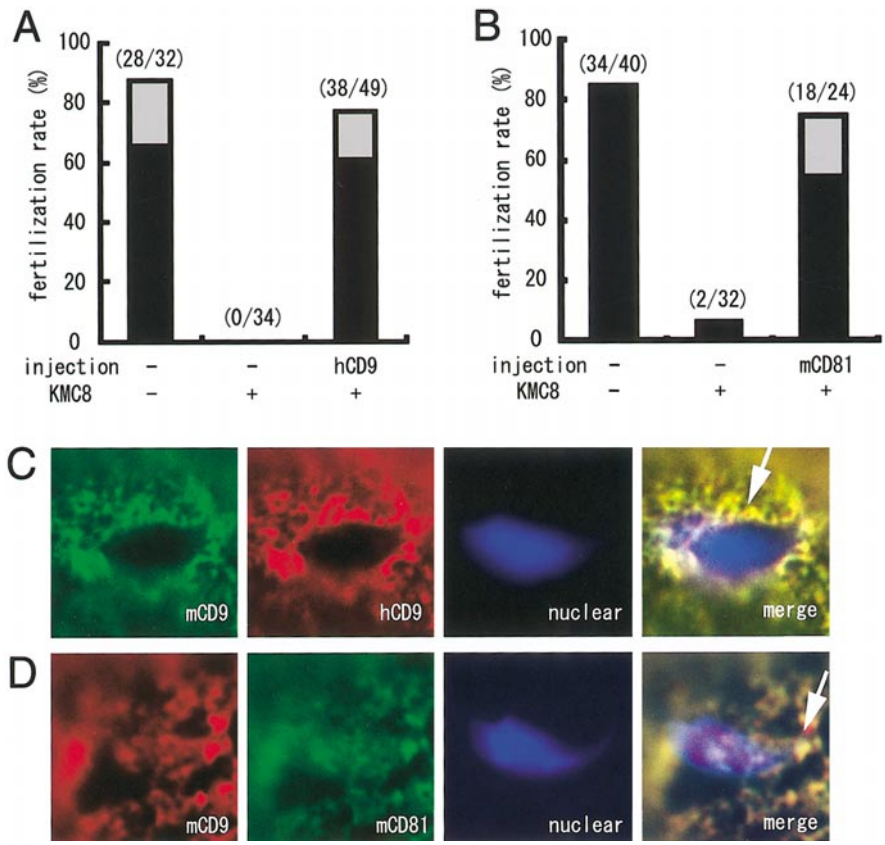

FIG. 4. Recovery of fertilization rate by expression of human CD9 or mouse CD81 in the presence of anti-mouse CD9 mAb, KMC8. Zona-free normal eggs and eggs overexpressing human CD9 and mouse CD81 were cultured for 30 min in the presence or absence of 50 μg/ml anti-CD9 mAb, KMC8, and then inseminated by adding the sperm to the culture medium. The fertilization rates of eggs overexpressing human CD9 (A) and mouse CD81 (B). The numbers of fertilized eggs in total eggs are noted above each column. The percentages of eggs at anaphase and that of eggs with second polar body are shown in gray and black, respectively. Experiments were done four (A) or three (B) times with more than seven eggs in each group. (C, D) Localization of human CD9 and mouse CD81 together with mouse CD9 on the egg microvilli. After insemination in the presence of anti-CD9 mAb, eggs overexpressing human CD9 and mouse CD81 were stained with the anti-mouse CD9 mAb (green) and the anti-human CD9 mAb (red) (C), or anti-mouse CD9 mAb (red) and the anti-mouse CD81 mAb (green) (D), and the surfaces were observed. Chromosomes of sperm (blue) were stained with Hoechst 33342. The right panel shows superimposed images of the three panels on the left. Human CD9 and mouse CD81 were colocalized with endogenous mouse CD9 surrounding the spermhead (arrows). The staining data shown are representative of all experiments.

overexpression of hCD9 or mCD81. As shown in Figs. 4A and 4B, in the presence of 50 μg/ml KMC8, zona-free eggs hardly fertilized, but the fertilization rates of eggs with hCD9 and mCD81–poly(A) RNA injection were restored to 78 and 75%, respectively. These results indicate that overexpression of hCD9 and mCD81 can rescue the fusibility blocked by the anti-CD9 mAb. Moreover, for the percentages of fertilized eggs with PB2 (black), the restored rates against that of control eggs, inseminated in the absence of an anti-mCD9 mAb, were 93 (hCD9) and 64% (mCD81), respectively (Figs. 4A and 4B). The eggs with the second polar body 2 h after insemination are considered to be eggs fertilized within 1 h after insemination because it takes more than 1 h from the time of sperm–egg fusion to completion of the second polar body formation. Therefore, in the rescue of fusibility blocked by an anti-mCD9 mAb, the activity of mCD81 was lower than that of hCD9 in accord with the results in Fig. 3. After insemination, eggs with hCD9- or mCD81–poly(A) RNA injection were stained with the corresponding antibodies, and the high levels of expression of exogenous proteins were detected (data not shown). As shown in Figs. 4C and 4D, overexpressed hCD9 and mCD81 molecules were colocalized with endogenous mCD9, which is possibly localized mainly in the microvilli (Kaji *et al.*, 2000), and they surrounded the sperm heads (shown by arrows).

DISCUSSION

In this report, we show that the exogenous mouse CD9 was abundantly expressed on egg plasma membrane by

poly(A)–RNA injection and the exogenous CD9 reversed the infertility of $CD9^{-/-}$ eggs. Therefore, this technique could be effective for analyzing the functional domain of CD9 and the function of other molecules expected to be associated with CD9 for participation in sperm–egg fusion. Unexpectedly, as shown in Fig. 2, the fertilization rate of $CD9^{+/-}$ eggs was lower than that of $CD9^{+/+}$ eggs, correspondent with the lower expression level of CD9, despite the normal number of pups born to a $CD9^{+/-}$ female mouse; the contradiction between *in vitro* and *in vivo* fertilization may the result of the different interaction times between eggs and sperm. In fact, even in $CD9^{-/-}$ eggs, the fertilization rate was increased *in vitro* when the coincubation time with sperm was longer (Miyado *et al.*, 2000; Kaji *et al.*, 2000). In this report, human CD9 compensated for the function of mouse CD9 at a high level in $CD9^{-/-}$ eggs. This is the first finding to suggest the contribution of hCD9 to human fertilization, and the research into human contraception that might be performed with mouse eggs using our method is very interesting. The fact that mCD81 can compensate for the function of mCD9 is also remarkable, though both the activity and the endogenous expression level on eggs were lower than those of mouse CD9. This result first directly suggests that tetraspanins can functionally substitute each other, and, in CD9-deficient mice, the substitution by other tetraspanin(s) may cause no abnormalities except female sterility. Moreover, although the inhibitory mechanism of fertilization by anti-mCD9 mAb is not clear, the antibody treatment did not make the eggs infertile, but the inhibition may be due to blocking against the interaction between mCD9 and other molecule(s), because the inhibition was overcome by overexpression of human CD9 and mouse CD81

Although the amounts of different molecules expressed by RNA injection are not comparable with each other strictly because they were visualized only by staining with proper antibodies, our results suggest that human CD9 and mouse CD81 can compensate for the function of mouse CD9 in sperm–egg fusion. The function of human CD9 and monkey CD9 as a coreceptor of diphtheria toxin and that of human CD81 as a receptor of Hepatitis C virus are not replaced by other tetraspanins (Nakamura *et al.*, 2000; Pileri *et al.*, 1998). These functions are strictly determined by the two or three amino acid residues between the conserved CCG motif and the fourth cysteine residue of the large extracellular loop domain (Hasuwa *et al.*, 2001; Higginbottom *et al.*, 2000). There are only five matches in amino acid residues between mCD9 and mCD81 in the region I152–P178 of mCD9; thus the functional replacement of CD9 with mCD81 in sperm–egg fusion may be determined by the three-dimensional structure of the molecules. The function and functional domain(s) of CD9 in sperm–egg fusion remain unclear, but in further experiments, the technique developed in this work may be a powerful tool with which to reveal not only the role of CD9 but also the other molecular mechanism in sperm–egg fusion.

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